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Carbon Nanotube-based Cholinesterase Biosensors for the Detection of Pesticides

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1. Introduction

Pesticides play an important role in the high productivity achieved in agriculture through the control of pests. However, the presence of pesticide residues and metabolites in food, water and soil currently represents one of the major issues for the environmental chemistry(Ongley, 1996; Smith and Gangolli, 2002; Lintellman et al., 2003). Pesticides are often very persistent with half-lives of decades and are transported over long distances by global circulation, and through run-off, find their way into aquatic systems. They are intentionally toxic, often towards non-target organisms. Three classes of pesticides have been problematic, namely organophosphates, carbamates and organochlorines. Organophosphate(OPs) pesticides obtain their toxicity from their ability to inhibit acetylcholinesterase (AChE), causing neurotoxicity (Fukuto, 1990). The presence of this enzyme in insects, birds, fish and all mammals give this class of pesticides enormous toxicity towards unintended targets. Carbamate pesticides are also cholinesterase inhibitors with a similar mechanism of action as organophosphate pesticides (Fukuto, 1990). An effective strategy for dealing with pesticide contamination in the environment has to commence with an assessment of the extent of the problem. Traditionally, chromatographic methods have been used to analyze the presence of compounds in environmental samples. These techniques are very powerful tools for monitoring toxic pesticides, but, they are expensive, time-consuming (involve extensive preparation steps), are not adapted for in situ and real time detection and require highly trained personnel. They are therefore unsuitable for screening of large volumes of samples, and due to their cost, developing countries do not readily have access to such methods.

Biosensors has attracted intensive research interest as a result of the need for cheap, fast and easy to use analytical tools that are able to provide real-time qualitative and quantitative information about the composition of a sample with minimum sample preparation (Dyk and Pletschke, 2011). Biosensors are analytical devices which utilize the sensitivity and selectivity of a bio-receptor attached onto the surface of a physical transducer. The cholinesterase (ChE) enzymes based biosensors have emerged as an ultrasensitive and selective technique for toxicity monitoring for environmental, agricultural, food or military

applications (Silvana and Marty, 2006). These devices are based on the inhibition of ChE by toxicants such as pesticides. The principal motivation for designing ChE biosensors for toxicity monitoring is to provide a reliable alternative to classical methods currently used in chromatographic methods. A successful ChE biosensor for toxicity monitoring should offer comparable or even better analytical performances than the traditional chromatographic systems. Ideally, such sensors should be small, cheap, simple to handle and able to provide reliable information in real-time without or with a minimum sample preparation. The use of the enzyme should also provide increased sensitivity and selectivity for the analyte of interest.

Electrochemical biosensors are currently among the most popular of the various types of biosensors. Carbon nanotubes (CNTs) are promising materials for sensing applications due to fascinating electronic and optoelectronic properties that are distinct from other carbonaceous materials and nanoparticles of other types (Balasubramanian and Burghard, 2006). Particularly, the properties of small dimensions, functional surfaces, good conductivity, excellent biocompatibility, modifiable sidewall, and high reactivity make CNTs have some overwhelming advantages in fabricating electrochemical sensors with high performances (Rivas et al, 2007). Moreover, CNTs have an outstanding ability to mediate fast electron-transfer kinetics for a wide range of electroactive species, such as AChE. CNT chemical functionalization can be used to attach almost any desired chemical species to them, which allows us to enhance the solubility and biocompatibility of the tubes. This has permitted the realization of composite electrodes comprising CNTs well-dispersed in an appropriate polymer matrix(Wang et al, 2003).

Generally, the replacement of ordinary materials by CNTs can effectively improve the redox currents of inorganic molecules, organic compounds, macrobiomolecules or even biological cells. Due to the well-defined structure, the chemistry stability and the electrocatalytic activity toward many substances, CNTs are also extensively used as the carrier platforms for constructing various electrochemical sensors. Herein, we present an overview of significant advances in the research and development of CNT-based ChE biosensors. We will discuss the different configurations and fabrication techniques of CNT-based biosensors with a special emphasis on the low-cost electrochemical biosensors and the approaches used for enzyme immobilization.

2. Inhibitory effect of pesticides on Cholinesterase

Many enzymes used for the detection of pesticides are inhibited by the pesticide and the extent of inhibition is correlated to the concentration of the analyte. Acetylcholinesterases are a class of enzymes that catalyze the hydrolysis of acetylcholine, an ester which is a neurotransmitter (Fukuto, 1990; Stenersen, 2004). The reaction catalyzed by AChE is: acetylcholine+H_2O→choline+acetate. Organophosphate and carbamate pesticides are designed to inhibit AChE and this enzyme has been mostly used in enzymatic detection of these pesticides. The inhibition of AChE by organophosphates takes place as a result of the phosphorylation of the serine residue in the active site of the enzyme. The hydroxyl group on the serine residue acts as an electrophile which attacks the nucleophilic phosphorus. The phosphorylated enzyme is highly stable and the hydrolysis of acetylcholine is blocked. In some cases, depending on the chemical structure of the pesticide, the phosphorylation, and thus the inhibition, may be irreversible (Fukuto, 1990). Where the pesticide is a

phosphorothionate ester with a P=S moiety rather that a P=O, these pesticides generally act as poor cholinesterase inhibitors, due to the low reactivity of the compound (Fukuto, 1990). An example of two phosphorothionate ester compounds is malathion or chloropyrifos. It would be expected that these compounds would be poor cholinesterase inhibitors. This can be overcome by chemically oxidizing the phosphorothionate ester compounds into its more active form prior to detection (Lee et al., 2002).

It is helpful for the development of a biosensor based on enzyme inhibition to know the structure of ChE enzyme and the mechanism of inhibition in order to better optimize several parameters which affect the degree of inhibition such as enzyme loading, incubation time, reaction time, concentration of substrate, pH and organic solvent (Arduini, 2010). The principal biological role of AChE is the termination of the nervous impulse transmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine. Early kinetic studies indicated that the active site of AChE contains two sub-sites, the esteratic and anionic sub-sites, corresponding respectively, to the catalytic site and choline-binding pocket (Gordon, 1976). The esteratic site contains a serine residue which reacts with the substrate and, also, with the organophosphates and carbamates. This site is similar in the multiple forms of AChE (Electrophorus, Torpedo, rat and chicken) and it is also located in the butyrylcholinesterase (BChE) enzyme. For this reason, it is possible to use several species of AChE and BChE enzymes to develop a ChE biosensor for insecticides.

The substrate concentration can affect the degree of inhibition. It was found that the inhibition level (%) increases with increasing of the substrate concentration in the case of pesticide inhibition when a saturating substrate concentration was used (Kok et al, 2002). Joshi et al. (2005) have used a concentration of acetylhtiocholine two times higher than the apparent Michaelis–Menten constant (K_m) for the determination of the maximum activity of AChE before and after the inhibition by the paraoxon which was selected as model pesticide. In the case of competitive inhibition, at high substrate concentrations, the inhibition effect is not observed since the substrate competes with the inhibitor. Another enzyme that is related to AChE is BChE which is also termed pseudocholinesterase (Andreescu and Marty, 2006). Both these enzymes have been used for the detection of pesticides in the environment, but use different substrates. AChE and BChE also differ in that AChE is inhibited by excess substrate whereas BChE is not. In the case of irreversible inhibition, the high substrate concentration can be chosen in order to have a higher output signal. For AChE biosensor a concentration of 1 mM acetylthiocholine was adopted (Arduini, 2006). In order to obtain higher sensitivity in the case of biosensor format for insecticides, acetylcholine or acetylthiocholine for AChE biosensor and butyrylcholine or butyrylthiocholine for BChE biosensor is highly suggested (Arkhypova, 2004).

For irreversible inhibition it is possible to achieve lower detection limits using longer incubation times; in fact, usually the degree of the enzyme inhibition increases with the incubation time until reaching a plateau (Kok, 2004). The incubation time is usually chosen as compromise between a sensitive measurement and a measurement carried out in a reasonable time (Dzyadevych, 2005). Usually, the incubation time should be not longer than 1 h because one of the biosensor advantage than i.e. HPLC should be the short time of analysis. In order to increase the sensitivity of the biosensor, it is better varying the enzyme loading than to use the incubation time longer than 1 h. In fact, for irreversible inhibition the degree of inhibition depends of the enzyme concentration.

The pH of the solutions containing substrates can affect the overall enzymatic activity since, like all natural proteins, enzymes have a native tertiary structure that is sensitive to pH;

denaturation of enzymes can occur at extreme pHs. It is well known that the enzyme activity is highly pH dependent and the optimum pH for an enzymatic assay must be determined empirically. It is best to choose a plateau region so that the pH should not have any effect on enzyme activity and will not interfere with the results obtained relative to the inhibition of the enzyme by the inhibitor. The activity of the immobilized acetylcholinesterase as a function of pH was studied between pH 2 and 9 and the activity of approximately 70% decreased at pH 2 in comparison with that at pH 7 (Stoytcheva et al, 2002). For the selection of the pH, it should be considered that certain enzymes have ionic groups on their active site and these groups must be in a suitable form such as the serine group in the catalytic site of ChE enzymes. The optimum pH for the free enzyme is pH=8. The acid pH should be avoided, in fact in the case of insecticide detection during wine fermentation (Suprun, 2005) it is necessary to adjust the pH towards neutral value.
The extraction of pesticides is carried out using organic solvents as reported in the official methods for pesticides detection, so it is important to choose an appropriate organic solvent to reduce the enzyme inactivation. To understand the possibility to use the organic solvents for insecticide detection with biosensor, their effect on ChE activity was investigated. Depending on the nature and the amount of organic solvent involved, the enzyme can be strongly inactivated when experiments are performed in these media. Thus, the choice of organic solvent needs to be considered as part of the method development in order to avoid undesirable effects. The effects of organic solvents have been shown to be quite variable and depend on the configuration in which the enzyme is employed. For example, the influence of acetonitrile, ethanol and DMSO on a cholinesterase sensor was studied using acetylthiocholine as substrate (Montesinos et al, 2001). An increase of the output current was noticed when working in 5% acetonitrile and 10% ethanol, resulting from partial deactivation of the enzyme. Therefore, the choice of an appropriate organic solvent is important to circumvent or minimize the enzyme inactivation.
AChE from different sources may vary in their reactivity to pesticide inhibition and sensitivity (limit of detection). Comparative studies have been performed with various wild-type AChE as well as some genetically modified enzymes. Of the wild-type enzymes, AChE from electric eel was more sensitive than AChE from bovine and human erythrocytes. In addition, greater sensitivity was achieved with the genetically modified AChE from Drosophylla melanogaster.

3. Pretreatment and dispersion of carbon nanotube

3.1 Pretreatment of carbon nanotube

CNTs possess interesting electrochemical properties and the improvement in the electron transfer is due to the curvature of the tubes that originate changes in the energy bands close to the Fermi level (Britto et al, 1999). The presence of pentagonal defects produce regions with charge density higher than those observed in the region of hexagonal graphite, either in planar or in tubular structures (Britto et al, 1999). Usually, CNTs should be pretreated in order to eliminate metallic impurities, and/or to improve the electron transfer properties and/or to allow further functionalization. The protocols are based on the oxidation of CNTs under different conditions. In all cases the ends and side-walls become rich in oxygenated functions, mainly carboxylic groups. Depending on how drastic is the treatment, it is possible not only to increase the density of oxygenated functions but also to break the tubes or even to shorten them(Rivas et al, 2007). Solutions of sulfuric, nitric, and hydrochloric

acids and, either concentrated or diluted, alone or mixed have been extensively used for activation of CNTs. In some cases, the pretreatments were based on a combination of different chemical and electrochemical protocols (Rivas et al, 2007).
Generally, CNTs exist as highly tangled ropes and are insoluble in almost all solvents, thus, greatly hindering their capacity of forming uniform and stable films. Therefore, the major problem on the promising applications of CNTs in electrochemical sensors is the immobilization of activated CNTs on the electrode surface. To overcome this deficiency, CNTs are firstly dispersed or dissolved in various solutions or suspensions and immobilized on the surfaces of various substrates by physical or chemical methods.

3.2 Solvent dispersion of carbon nanotube

The most widely protocols for fabricating CNT-based electrochemical sensors involve the dispersing of CNTs in a certain solvent with sonication after purification and activation pretreatments of CNTs, followed by dropping the resultant suspensions on the electrode surfaces and allowing to dry (i.e., the casting methods). Among the reported solvents, N,N-dimethylformamide (DMF) is the most extensively used polar solvent and more than half of the papers deal with CNT-based electrochemical sensors using DMF as the dispersing solvent (Xu et al, 2004). The other solvents, which have been used to prepare CNT suspensions, include water (Guo et al, 2005), acetone (Wu et al, 2002), ethanol (Qu et al, 2004) and even toluene (Lefrant et al, 2004). Compared with other approaches, the solvent-dispersing methods of CNTs inevitably suffer from some disadvantages, such as low solubility, low stability and low exfoliation efficiency, due to the rather weak interactions between these solvents and CNTs.

3.3 Additives -assisted dispersion of carbon nanotube

The preparation of an electrochemical sensor modified with a CNT-dispersion basically consists of casting the electrode, usually glassy carbon or gold, with a drop of the given dispersion, followed by a drying step under different conditions. To improve the solubility and stability of CNTs in their suspensions, various additives are added into solvents to assist the dispersion of CNTs, such as surfactants, polymers, proteins and cyclodextrins.
Dihexadecyl hydrogen phosphate (DHP) consists of two hydrophobic tails and a dissociable phosphate group. Based on the hydrophobic interactions between the hydrophobic tails of DHP and the sidewall of the nanotubes as well as the possible interactions between the phosphate groups on DHP and the oxygen-containing groups on acid-treated CNTs, MWNTs were able to disperse in the aqueous suspension of DHP with sonication, resulting in the formation of a stable and homogeneous aqueous suspension of DHP and CNTs (Wu et al, 2003 and 2004; Wang et al, 2004). Sodium dodecyl sulphate (SDS) has been widely used to prepare stable suspensions of purified SWNTs in water with the aid of sonication (Abatemarco, 1999). It is suggested that the encapsulation of SWNTs in SDS micelles and the repulsive interactions between negatively charged SDS micelles account for the stable suspension of SWNTs in solution. The suspension of CNTs in cetyltrimethylammonium bromide (CTAB) aqueous solution was also reported (Cai and Chen, 2004). Nafion has been used extensively for the modification of electrode surfaces and for the construction of amperometric biosensors because of the unique ion-exchange, discriminative, and biocompatibility properties (Fortier et al, 1992; Fan et al, 1992). Nafion contains two different regions: the hydrophobic polymer backbone and the ionized hydrophilic sulfonate groups

outside the hydrophobic region. This special amphiphilic structure makes Nafion bear the capacity of combining with CNTs by hydrophobic interactions between the hydrophobic backbone of Nafion and the sidewall of CNTs as well as dispersing them in solutions by the hydrophilic groups.

As a kind of special amphiphilic biomacromolecues, proteins are proved to be capable of dispersing CNTs in water. It was found that SWNTs are naturally protein-affinitive in an aqueous ferritin solution (Lin et al, 2004). The conjugation can be further enhanced and stabilized in the presence of a coupling agent for amidation to promote the formation of covalent linkages.

Cyclodextrins (CDs) are cyclic oligosaccharides and contain a relatively hydrophobic central cavity and hydrophilic outer surface. CDs were able to adsorb onto the surface of nanotubes via van der Waals forces. The soft cutting of SWNTs by CDs was observed, indicating the use of CDs for the chemical manipulation and processing of CNTs (Chen et al, 2001).

Positively charged polyethyleneimine (PEI) has also been extensively employed as an efficient additive for the dispersion of CNTs in aqueous solutions. The resulting PEI-CNT composite was proved to have good stability and biocompatibility. Based on the reactivity of amino groups on PEI, the noncovalent or covalent modification of CNTs by PEI provides a simple approach to the further surface functionalization of CNTs by quantum dots (Shan and Gao, 2006), metal nanoparticles (Hu et al, 2006 and 2007). PEI-functionlized CNTs are a useful nanocomposite in electromistry and electroanalytical chemistry. Similar to PEI, Polylysine is an efficient dispersing agent of MWCNT because of the large amount of amine residues that facilitate the interaction with the MWCNT(Rivas et al, 2007). It was found that the mild sonication of MWNTs in aqueous poly(diallyl dimethyl ammonium) chloride (PDDA) resulted in a significant improvement of CNT dispersibility and enhanced their adhesion to Au and Si substrates (Mamedov et al, 2002).

Chitosan (CHIT) displays excellent film-forming ability, high water permeability, good adhesion, and susceptibility to chemical modifications due to the presence of reactive amino and hydroxyl functional groups. The stable dispersion of CNTs was prepared in the acidic aqueous solutions of CHIT with the help of sonication (Jiang et al, 2004). It was demonstrated that CHIT might be adsorbed onto CNTs and form a special CHIT-CNT system, which can be precipitated from the solutions by the addition of concentrated salts or the adjustment of solution acidity. The selective interaction between CHIT and CNTs also provided a possible approach for separating CNTs from carbonaceous impurities.

4. Modification of CNT-based electrodes by metallic nanoparticles

Transition metals such as gold, platinum, palladium, copper, silver and nickel are well-known for their high catalytic activity. Nanoparticles (NPs) made from these transition nanoparticles have been widely utilized to enhance the performances of electrodes made of carbonaceous materials, and, in particular, to increase their sensitivity towards a specific analyte. Because nanoparticles can provide a larger surface area and be easily modified with a wide range of biomolecules they enable the fabrication of biosensors with a plethora of sensing possibilities.

Both single-walled carbon nanotubes (SWCNTs) and multiwalled carbon nanotubes (MWCNTs) have been modified with different metal nanoparticles via the adsorption of preformed nanoparticles, or via electrodeposition from metal salt solutions. The latter

method is especially attractive since it allows precise control over the degree of modification in a reproducible manner (2004; Day et al, 2005). By controlling the duration and magnitude of the applied potential in conjunction with the concentration of the salt used, different sizes and densities of such particles can be obtained (Day et al, 2005). Among those nanoparticles that have been commonly deposited on carbon nanotubes, gold nanoparticles (GNPs) have been paid much interest. It has been reported that CNTs/GNPs composites could be synthesized through citrate reduction or were electrochemical deposition of GNPs on CNTs via in situ reduction of $HAuCl_4$ by $NaBH_4$ (Xiao et al, 2008). Choi et al. reported that GNPs could be generated on single-walled carbon nanotubes (SWCNTs) through direct redox reactions by immersing SWCNTs in a solution of Au^{3+} ions (Choi et al, 2002). Another reports demonstrated that GNPs was deposited on the side wall of multiwalled carbon nanotubes(MWCNTs) by one-step reaction with $NaBH_4$ as reductant and stabilized with sodium citrate in aqueous solution to form MWCNTs-GNPs nanocomposite(Du et al, 2010). During this synthesis process, GNPs could be self-coated on the MWCNTs to form uniform nanocomposites. The formed MWCNTs-Au nanocomposites offered an extremely hydrophilic surface for biomolecule adhesion, leading to a stable acetylcholinesterase biosensor. Due to the excellent conductivity of the nanocomposites, the immobilized AChE showed favorable affinity to acetylthiocholine (ATCl) and could catalyze the hydrolysis of acetylthiocholine to form thiocholine, which was then oxidized to produce a detectable and fast response. Using malathion as a model compound, the inhibition of malathion was proportional to its concentration ranging from 1.0 to 1000 ng mL^{-1} and from 2 to 15 μgmL^{-1}, with a detection limit 0.6 $ngmL^{-1}$.

5. Immobilization of cholinesterase onto CNT-based electrodes

The selectivity and sensitivity of CNT-modified electrodes can be improved through the immobilization of enzymes. In such electrodes, the CNTs mainly serve as transducers, communicating the signal effectively from the active enzyme centers to the substrate. After the choice of transducer, the enzyme immobilization is an important step in the biosensor design. The development of biosensors based on immobilized enzymes came about to solve several problems such as loss of enzyme (especially if expensive), maintenance of enzyme stability and shelf life of the biosensor, and additionally to reduce the time of the enzymatic response and offer disposable devices which can be easily used in stationary or in flow systems. In order to increase the storage stability in a dry state, which is a key point to commercialize the ChE biosensor, the immobilization should maintain the enzyme activity also in a dry state for several days. To do this, several types of immobilization techniques relying on noncovalent, covalent bonding or covalent cross-linking of the enzyme were investigated in order to obtain sensitive and stable ChE biosensors. The noncovalent approach, which has a negligible effect on the activity of the enzyme, can be subdivided into adsorption, entrapment and microencapsulation techniques. In the first technique, the enzyme is attached with the help of a binder or an ion-exchange resin onto CNT-modified electrodes. Alternatively, the enzyme can be immobilized using surface groups of self-assembled monolayers or Langmuir–Blodgett (LB) films. In the entrapment method, a (electro)polymerization reaction is carried out during which the enzyme is incorporated into the resulting polymer matrix. The encapsulation method makes use of hydrogels or sol-gels to immobilize the enzyme.

5.1 Enzyme adsorption

The physical immobilization such as adsorption is one of the simple procedure to immobilize the biocomponent onto the transducer (Bonnet, 2003). AChE was immobilized by adsorption on screen printed electrodes modified with multiwall carbon nanotubes (MWCNTs). In this way, some µL of AChE solution were dropped on the MWCNT modified electrode surface and allowed to evaporate at room temperature under a current of air. The electrode was then rinsed twice with buffer to remove the loosely adsorbed enzyme molecules on MWCNTs (Joshi, 2005). This was an important step to avoid the leakage of the enzyme during the measurement. One of the most sensitive biosensors was developed by immobilizing AChE via physical adsorption in nanostructured carbon matrix as reported (Sotiropoulou and Chaniotakis, 2005). The obtained biosensor is very stable and has very low detection limit for dichlorvos at picomolar levels. This promising result was attributed to the properties of the activated carbon to preconcentrate the insecticides and the hyperactivity of enzyme within the nanopores. Nevertheless, the immobilization of enzymes via adsorption faces several problems, such as the low quantity of adsorbed enzyme, leaching of the enzyme and so on. Some of these limitations can be overcome by adsorbing enzymes onto CNT-modified electrodes decorated with metallic nanoparticles, such as Pt-NP. By subsequently depositing a Nafion film onto the electrode, it is possible to reduce leaching of the enzyme and to improve the stability of the biosensor.

5.2 Self-assembling immobilization

During the acid treatments of CNTs, the negatively charged carboxyl groups produce, providing two methods for the direct self-assembling immobilization of CNTs on electrode surfaces. In the first method, CNTs are adsorbed onto electrode surfaces via the electrostatic interactions between negatively charged carboxyl groups on CNTs and the positively charged species on the electrode surfaces. The second method is the covalent bonding of CNTs to cysteamine self-assembled monolayer (SAM) modified gold electrodes via the reaction of carboxyl groups on CNTs and amino groups on cysteamine SAM in the presence of coupling reagents (Liu et al, 2000). As-grown nanotubes were first cut into short pipes and thiol-derivatized at the open ends by chemical methods. The ordered assembly of SWNTs was then made by their spontaneous chemical adsorption to gold via Au–S bonds. The nanotubes were found to be organized on gold, forming a self-assembled monolayer structure with a perpendicular orientation.

A recent approach to immobilize AChE was proposed based on the layer-by-layer (LbL) self-assembly of oppositely charged polyelectrolyte and enzyme onto previously functionalized CNT-modified electrode (Liu et al, 2006). In one case, the negative charges were originated onto the surface of carbon nanotubes by adsorbing a pyrene derivative, which serves as platform for further assembling of poly(diallyldimethylammonium) (PDDA) and polystyrene sulfonate (PSS).

For the detection of carbaryl pesticides, an amperometric biosensor is fabricated based on consectively adsorption of the PDDA modified single walled carbon nanotubes (PDDA-SWCNTs) and acetyl cholinestrase onto the surface of glassy carbon electrode(Firdoz et al, 2010). The optical intensity of UV/vis spectra increased with the number of layers, indicating the build up of a multilayer coating on the electrode. The biosensor from $[(\text{PDDA-SWCNTs})/\text{AchE}]_5$ coated glassy carbon electrode showed good sensitivity and stability towards the monitoring of carbaryl pesticides in water with the detection limit of 10^{-12} g.L^{-1} and recovery of 99.8 ±2.7% to 10^{-10} g.L^{-1}.

A highly sensitive flow injection amperometric biosensor is fabricated for the detection of organophosphate pesticides based on self-assembled AChE on a carbon nanotube-modified glassy carbon electrode. The MWCNTs was initially treated with NaOH in order to assume a negative charge and then was dipped into a solution of cationic PDDA which leads to the adsorption of positively charged polycation layer (MWCNTs-PDDA). After the negatively charged AChE was adsorbed on MWCNTs-PDDA to obtain MWCNTs-PDDA-AchE, another PDDA layer was absorbed in order to avoid the leakage of AChE from the electrode surface, resulting in sandwich structure of PDDA/AChE/PDDA(Liu et al, 2006). The unique sandwich-like structure (PDDA/AChE/PDDA) on the MWCNTs surface formed by self-assembling provides a favorable microenvironment to keep the bioactivity of AChE. The electrocatalytic activity of MWCNTs leads to a greatly improved electrochemical detection of the enzymatically generated thiocholine product, including a low oxidation overvoltage (+150 mV), higher sensitivity, and stability. The developed PDDA/AChE/PDDA/ MWCNTs/GC biosensor integrated into a flow injection system was used to monitor organophosphate pesticides and nerve agents, such as paraoxon. Under the optimal conditions, this biosensor allows a low detection limit of paraoxon equal to 0.4×10^{-12} M with a 6-min inhibition time. The biosensor had excellent operational lifetime stability with no decrease in the activity of enzymes for more than 20 repeated measurements over a 1-week period.

5.3 Covalent attachment

The adsorption method normally yields a random distribution of the enzymes on the electrode. However, direct anchoring of the enzymes to the carbon framework becomes feasible if the covalent immobilization approach is used. In addition, it often enables direct electron transfer to the active center of the enzyme. One of the most used types of enzyme immobilization is the chemical immobilization by means of cross-linking with glutaraldeyde. This method confers to the biosensor high working stability even if there is usually a decrease of the enzymatic affinity towards its substrate due to the distortion of the enzyme structure (Arduini et al, 2006 and 2007; Vakurov, 2004). An example of chemical immobilization is based on a non-conducting polymer electrosynthetized onto the electrode after the enzyme was immobilized by crosslinking with glutaraldehyde (Curulli et al, 2001; Suprun et al, 2004). ChE immobilization was able to carry out by cross-linking method with glutaraldehyde vapour (Arkhypova et al, 2003, 2004 and 2008). It was reported a ChE membrane formed on the electrode with ChE, Nafion and glutaraldehyde (Suprun et al, 2005; Ivanov et al, 2003). In this case, it was found that the use of albumin bovine serum at 3% increases the enzyme stability (Arduini et al, 2006; Laschi et al 2007).

5.4 Electropolymerization

The polymerization of various monomers in the presence of dispersed CNTs in solutions by electrochemical methods has been employed for the immobilization of CNTs on the electrode surface. In this process, CNTs were enwrapped in polymers during the electropolymerization process in the form of counter ions or dopants. A marvelous method was proposed for the dispersion and immobilization of CNTs on the electrode surface by water-soluble alizarin red S (Wu et al, 2004). Electropolymerization is also an attractive and well-controlled method for immobilizing enzymes onto electrodes. In this methodology, the enzyme is mixed with a monomer which is electropolymerized at a GCE or a metal

electrode, whereupon the enzyme becomes embedded into the polymer matrix. The incorporation of the enzyme into the matrix is often promoted through electrostatic interactions. Numerous enzymes have been incorporated into electropolymerized films(Bartlett and Cooper, 1993). In many cases conductive polypyrrole (PPy) has been used as a polymer matrix. This choice relates to the fact that pyrrole can be electropolymerized at low oxidation potentials in aqueous solutions at neutral pH, which is compatible with a wide range of biological molecules. Polypyrrole has proven effective at electrically wiring the enzymes and CNTs to the underlying electrode. During the fabrication of such biosensors, CNTs bearing carboxylic groups are often used due to their ability to function as an anionic dopant in the matrix.

Recently, a simple method to immobilize AChE on PPy and polyaniline (PAn) copolymer doped with multi-walled carbon nanotubes (MWCNTs) was proposed(Du et al, 2010). The synthesized PAn-PPy-MWCNTs copolymer presented a porous and homogeneous morphology which provided an ideal size to entrap enzyme molecules. The surface hydrophilicity was improved greatly after forming a complex structure instead of a separate layer. It provided an excellent environmental and chemical stability around the enzyme molecule to stabilize its biological activity to a large extent, resulting in a stable AChE biosensor for screening of organophosphates exposure. MWCNTs promoted electron-transfer reactions at a lower potential and catalyzed the electro-oxidation of thiocholine, thus increasing detection sensitivity. Based on the inhibition of OPs on the AChE activity, using malathion as a model compound, the inhibition of malathion was proportional to its concentration ranging from 0.01 to 0.5 μg/mL and from 1 to 25 μg/mL, with a detection limit of 1.0 ng/mL. Advantages of the electropolymerization approach include the good control over the film thickness and the ability to selectively attach biomaterials onto nanoscale electrode surfaces. The developed biosensor exhibited good reproducibility and acceptable stability.

5.5 Encapsulation

The sol-gel and hydrogel have been widely used in recent years to immobilize biomolecules (e.g., enzymes) for constructing electrochemical biosensors because of their easy fabrication, chemical inertness, thermal stability and good biocompatibility. It was reported that the immobilization of ChE by encapsulation in sol-gel prepared by tetramethoxysilane (TMSO) and methyltrimethoxysilane (MTMSOS) showed in both cases a storage stability of several months (Anitha et al, 2004). However, the lack of electrochemical reactivity and the poor conductivity of these materials greatly hinder their promising applications. Therefore, carbon nanotube has been widely incorporated into the sol-gel or hydrogel matrix. A typical procedure for preparing CNT-based hydrogel or sol-gel consists of the dispersion of CNTs in solvents, the mixing of the CNT suspensions with the hydrogel or the sol-gel and finally the casting of the resultant matrix containing the immobilized enzyme on the electrode surfaces. CNT acted as both nanometer conducting wires and catalysts, which can effectively promote electron transfer between enzymes and the electrode surface. The main advantage of the encapsulation process is that the entrapped species often preserves its intrinsic bioactivity. Additionally, such sensors exhibit enhanced sensor response, due to an increase in the surface area as well as an improvement in the electrical communication between the redox centers of the hydrogel or the sol-gel-derived matrix and the electrode. Apart from hydrogels and sol-gels, Nafion has also been found to be useful when

fabricating composite electrodes. A broad range of enzymes has been successfully immobilized onto CNT-incorporated redox hydrogels to yield sensitive biosensors (Joshi, 2005). These CNT-based sol-gel electrochemical biosensing platforms were demonstrated to possess both the electrochemical characteristics of CNTs and the role of sol-gel for eliminating byproducts. In contrast to the conventional sol-gel or CNT-based electrochemical sensors, the electrochemical response of these electrodes can be conveniently tuned from that of conventional scale electrodes to that of microelectrodes by just varying the content of MWNTs in the composites. A sensitive and stable amperometric sensor has been devised for rapid determination of triazophos based on efficient immobilization of AChE on silica sol-gel film assembling MWNTs (Du et al, 2007). Under optimum conditions, the inhibition of triazophos was proportional to its concentration from 0.02 μM to 1 μM and from 5 μM to 30 μM, with a detection limit of 0.005 μM.

6. Practical concerns

The detection of pesticides is essential for the protection of water resources and food supplies. The designed biosensor should be sensitive enough to decrease the threshold detection as low as possible (Villatte et al., 1998 and Sotiropoulou et al., 2005). In addition, it should be selective towards the target analyte or class analytes. Before the benefits of enzymatic methods can be transferred from the laboratory to the field, it is important to stress that in the case of real samples the ChE biosensor is not a selective system because organophosphorus and carbamic insecticides and some other compounds have an inhibition effect on ChE. It has been demonstrated that an enzyme such as AChE is inhibited by organophosphate and carbamate pesticides by a similar mechanism of action but with different inhibition degree (Fukuto, 1990). This makes ChE biosensors unable to correctly differentiate and identify particular analytes, so the selectivity for measuring ChE inhibitors is very poor (Schulze et al., 2003 and Luque de Castro and Herrera, 2003). Therefore, ChE biosensors are mainly attractive for measuring the total toxicity of the sample, rather than a specific inhibitor. In fact, this behavior can be a disadvantage because other techniques are required in order to evaluate which inhibitor is present. Therefore, little success has been realized through real practical applications and commercialization of these devices for solving real world problems despite a significant amount of scientific research dedicated to ChE biosensors. Nontheless, this aspect can be also an advantage taking in consideration that this system is a screening method. Biosensors can be very useful tool to understand the presence of possible toxic compounds able to inhibit the ChEs, and only the samples in which the inhibition is observed will be measured by the reference method with a relevant saving in terms of time and cost of analysis (Dzydevych et al, 2002).

Further improvement in sensitivity and selectivity can be obtained with the use of sensitive multienzymes which allow discrimination between the insecticides and other interferences. Enzymes extracted from different sources have different sensitivities and selectivities toward pesticides. For instance, the AChE extracted from the Drosophila melanogaster is 8-fold more sensitive than the AChE from the Electric eel (Tsai and Doog, 2005). Moreover, advances in molecular biology have made possible engineering of more sensitive and selective ChE with individual sensitivity patterns towards a target inhibitor. Recombinant AChEs have been undertaken to increase the sensitivity of AChE to specific organophosphates and carbamates using site-directed mutagenesis and employing the

enzyme in different assay formats (Schulze et al, 2003). It was reported that an array of multienzyme biosensors constructed with four immobilized AChEs (wild type and three recombinant mutants) allowed discrimination of malaoxon and parathion in a binary composite mixture and enabled detection of 11 out of 14 organophosphate and carbamate pesticides (Bachmann et al., 2000 and Schulze et al., 2005).

ChE biosensors have great application potentials in environmental and food matrices, public safety and military/antiterrorism. Most ChE biosensors designed for practical applications use immobilized enzyme. However, as applied to inhibitor determination, the practical application of immobilized ChE has a significant limitation. The inhibition results in a decrease of the ChE activity so that repetitive use of the same biosensor without enzyme reloading or reactivation is limited. The solution to this problem is to employ single use disposable electrodes. These are usually prepared by screen-printed technology which allows mass production with significant reduction in the price per electrode.

The most studied pesticides are paraoxon, dichlorvos, diazinon, aldicarb and carbofuran. Paraoxon is commonly used as a model example for ChE inhibition. Some pesticides have nearly no or little inhibitory effect on ChE in their pure form. In this case, detection is still possible by oxidizing them to oxon forms, which are much more toxic. The typical example is the case of parathion, and its corresponding oxon form, paraoxon. In some cases, oxidation and detection of these pesticides has been improved with the use of a genetically modified mutant ChE enzyme (Schulze et al., 2004). Anatoxin-a(s) is a natural organophosphate which irreversibly inhibits AChE, similar to organophosphorus and carbamate pesticides. Due to the difficulty to detect this compound using classical analytical chemistry methodologies, research efforts have been directed toward the use of ChE biosensors, which allow detection of anatoxin-a(s) at very low concentrations (detection limit of 5×10^{-10}M) (Vilatte et al., 2002).

The superior electrocatalytic activity of CNT-based electrodes has sparked an explosive amount of research directed at using CNTs for electrochemical biosensing. In fact, a range of molecules can be easily oxidized at low potentials at CNT-based electrodes. Even if such electrodes are equipped with analyte-specific recognition units such as enzymes, they are still vulnerable to other electroactive compounds that can also be oxidized at these low potentials. Thus, for the assessment of a CNT-based biosensor, it is of utmost importance to carefully consider the interferents involved in the sample under consideration. The optimal composition of the biosensor is a trade-off between the various device parameters. A low amount of immobilized enzyme provides only a limited concentration range where the response is linear, whereas a large amount of enzyme could reduce the electrochemical activity of the CNTs. While direct immobilization of the enzyme without a matrix would be ideal for obtaining sensitive responses, such electrodes are prone to leaching of the enzyme. This loss and the subsequent reduction in sensitivity and reproducibility can be largely avoided by electropolymerized matrices.

In enzymatic detection methods, an initial concentrating step of the target analyte by liquid–liquid or solid-phase extraction methods has not been commonly used for further improvement of the sensitivity of detection. Yet, Marchesini et al. (2005) reported an increase in the limit of detection of 40 times where solid-phase extraction was used, although in this case the biorecognition element was not an enzyme but an antibody. It is expected that such methods could be applied to enzymatic detection to improve sensitivity, but may affect the portability of the method.

7. Conclusion

The most important challenge in the development of ChE biosensors for practical applications is the transfer of these devices from pristine research laboratory conditions to real-life and commercial applications. In this direction, some critical parameters such as enzyme stability, reliability and selectivity still have to be improved. This review highlighted the analytical parameters that should be investigated in order to increase the assay sensitivity using inhibition biosensors. The knowledge of the type of inhibition allows thus to optimize in a fast way the biosensor in order to increase the performance of the system and also to reduce the interferences. CNTs have been demonstrated to be an excellent material for the development of electrochemical biosensors. The incorporation of CNTs within composites offers the advantages of an easy and fast preparation, and represents a very convenient alternative as a platform for further design of biosensors with the improved performance. Considerable progress in genetic engineering allows for the production of more selective and sensitive ChEs. The design of each sensor containing a different immobilized enzyme (wild type and mutants ChEs extracted from different sources) could allow sensitive detection and differentiation of multianalyte mixtures. In addition, automated and continuous systems have been developed for measuring ChE inhibitors in flow conditions by a computer controlled-programmable valve system which allows reproducible pumping of different reagents including buffers, substrate and inhibitor solutions, reactivating agents and real samples. The combination of the unique properties of CNTs with the powerful recognition properties of sensitive multienzymes and the known advantages of the automated and continuous systems represents a very good alternative for the development of compact and portable devices able to address future biosensing challenges in environmental monitoring and security control, among others.

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A biosensor is a detecting device that combines a transducer with a biologically sensitive and selective component. Biosensors can measure compounds present in the environment, chemical processes, food and human body at low cost if compared with traditional analytical techniques. This book covers a wide range of aspects and issues related to biosensor technology, bringing together researchers from 12 different countries. The book consists of 20 chapters written by 69 authors and divided in three sections: Biosensors Technology and Materials, Biosensors for Health and Biosensors for Environment and Biosecurity.

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